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FINAL REPORT

Examination of KnockOut Mutants for Sensitivity to Phloroglucinol

Progress relates to Deliverable #1: Identification of the proteins involved in export of phloroglucinol and determination of whether overexpression of these proteins increases the concentration and yield of microbe-synthesized phloroglucinol.

Using transcriptome analysis, candidate genes were identified that may be involved in the export of phloroglucinol from the inside of the *Escherichia coli* catalyst to the culture medium. Gene expression in non-phloroglucinol synthesizing *E. coli* W3110 serA(DE3)/pBC1.146 was compared with gene expression in phloroglucinol synthesizing *E. coli* W3110 serA(DE3)/pJA3.131A. For both strains, cells were grown in 2-L fermentation vessels under identical conditions. Following transcriptome analysis, an extensive list of genes were identified whose expression changed substantially when phloroglucinol was synthesized by the cells. (Please see Progress Report 1). In order to gauge the importance of each gene product on cellular sensitivity to phloroglucinol, plans were laid to knock out the genes whose expression had increased in the presence of phloroglucinol. To determine which gene knock-outs should be prepared, genes that were upregulated at least two-fold were examined to determine if they were membrane proteins. From the list of upregulated genes, 41 genes were identified either as membrane proteins or putative membrane proteins (Table 1).

Because the number of candidate genes was substantial, prioritizing which knock-outs should be prepared first was difficult. To aid this process, a decision was made to look at additional transcriptome data. Under research funded by a MURI grant, *E. coli* cells cultured in shake flasks were exposed to phloroglucinol that was added to the culture medium. Comparison of the transcriptome data from these cells was compared to similar data for cells grown under identical conditions that were not exposed to phloroglucinol. Note that the MURI funded experiment examines transcriptional differences that occur when the cells are challenged with phloroglucinol outside the cell while the SBIR funded experiment examines the differences when the cells are challenged with phloroglucinol inside the cell. Thirty-seven genes corresponding to membrane proteins or putative membrane proteins were upregulated at least two-fold when phloroglucinol was added to the medium (Table 2).

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Table 1. Genes encoding membrane proteins upregulated at least two-fold in *E. coli* W3110 serA(DE3)/pJA3.131A under fermentor-controlled conditions

Gene	Description
yabN	putative transport protein
yacH	putative membrane protein
acrA	Acridine efflux pump
gltL	ATP-binding protein of glutamate/aspartate transport system
b0655	putative periplasmic binding transport protein
ybeX	putative transport protein
kdpB	ATPase of high-affinity potassium transport system
b0829	putative ATP-binding component of a transport system
b0830	putative transport protein
b0831	putative transport system permease protein
b0832	putative transport system permease protein
artJ	arginine 3rd transport system periplasmic binding protein
artM	arginine 3rd transport system permease protein
artQ	arginine 3rd transport system permease protein
artI	arginine 3rd transport system periplasmic binding protein
artP	ATP-binding component of 3rd arginine transport system
cydC	ATP-binding component of cytochrome-related transport
sapF	putative ATP-binding protein of peptide transport system
sapD	putative ATP-binding protein of peptide transport system
sapB	homolog of <i>Salmonella</i> peptide transport permease protein
sapA	homolog of <i>Salmonella</i> peptide transport periplasmic protein
b1451	putative outer membrane receptor for iron transport
b1601	putative transport protein
spy	periplasmic protein related to spheroblast formation
b2074	putative membrane protein
yegN	putative transport protein
bcr	bicyclomycin resistance protein; transmembrane protein
ompC	outer membrane protein
hisP	ATP-binding component of histidine transport
cysA	ATP-binding component of sulfate permease A protein
cysW	sulfate transport system permease W protein
acrD	multidrug transport protein
b3051	putative membrane protein
yhcD	putative outer membrane protein
yieO	putative transport protein (MFS family)
trkA	transport of potassium
yicM	putative transport protein
wzzE	putative transport protein
yjeP	putative periplasmic binding protein
ytfL	putative transport protein
yjhB	putative transport protein

Table 3. Common membrane genes upregulated at least two-fold in both experiments

Gene name	Description
acrD	multidrug resistance protein
b1451 (yncD)	putative outer membrane receptor for iron transport
b3051 (yqiK)	putative membrane protein
bcr	bicyclomycin resistance protein
trkA	transport of potassium
yacH	putative membrane protein
yegN	multidrug export protein

When the lists of overexpressed genes encoding membrane proteins from the two different experiments were compared, seven genes were found in common (Table 3). These genes were among the first given the highest priority for creation of knockout strains. Also given highest priority were *acrAB*, *yieO*, *yicM*, *yjhB*, *yjeP*, and *ytfL*, which were all identified in the fermentation experiment.

Inactivation of the *E. coli* chromosomal genes was carried out using a protocol that relies on the lamda recombinase system. Initial attempts to inactivate each gene directly in *E. coli* W3110 *serA*(DE3) were unsuccessful. Therefore, a two-step procedure was used in which the gene knockout was first generated in *E. coli* BW25113, which was confirmed via PCR. In a second step, P1 transduction was used to transfer the mutation from BW25113 into W3110 *serA*(DE3). PCR was again used to confirm the location of the knockout in the genomic DNA. Of the initial genes targeted, only *yegN* was problematic. Work continues on preparation of the *yegN* single knockout strain.

Each of the single knockout strains prepared was transformed with plasmid pJA3.131A, the *phlD*-containing plasmid. The resulting strains were then cultured in minimal salts medium containing glucose and varying concentrations of phloroglucinol. Cell density was then measured after 12 h of culturing (Table 4). The objective was to find a strain possessing a single knockout that was more sensitive to phloroglucinol, particularly at lower phloroglucinol concentrations. Unfortunately, none of the single knockout strains was more sensitive to phloroglucinol than the control strain W3110 *serA*(DE3)/pJA3.131A.

Table 4. OD600 of *E. coli* W3110 serA(DE3)/pJA3.131A and single knockout mutants in glucose minimal salts media containing varying concentrations of phloroglucinol 12 h after inoculation

	Phloroglucinol concentration (g/L)					
	0	0.5	1.0	2.0	4.0	8.0
W3110serA(DE3)/pJA3.131A	0.67	0.61	0.61	0.15	0.047	0.051
W3110serA(DE3)acrD/pJA3.131A	0.65	0.64	0.48	0.11	0.047	0.05
W3110serA(DE3)bcr/pJA3.131A	0.59	0.59	0.43	0.10	0.048	0.052
W3110serA(DE3)acrAB/pJA3.131A	0.64	0.64	0.47	0.10	0.034	0.039
W3110serA(DE3)yjhB/pJA3.131A	0.71	0.77	0.68	0.22	0.031	0.032
W3110serA(DE3)ytfL/pJA3.131A	0.66	0.69	0.67	0.29	0.028	0.039
W3110serA(DE3)yhcD/pJA3.131A	0.65	0.64	0.64	0.48	0.03	0.03
W3110serA(DE3)yicM/pJA3.131A	0.59	0.58	0.61	0.51	0.035	0.035
W3110serA(DE3)yieO/pJA3.131A	0.64	0.61	0.61	0.47	0.033	0.034
W3110serA(DE3)yncD/pJA3.131A	0.42	0.47	0.47	0.31	0.025	0.028
W3110serA(DE3)yacH/pJA3.131A	0.59	0.58	0.62	0.44	0.026	0.031
W3110serA(DE3)yqiK/pJA3.131A	0.54	0.53	0.63	0.38	0.024	0.030
W3110serA(DE3)trkA/pJA3.131A	0.002	0.008	0.009	0.011	0.013	0.017

To date, 30% of the gene candidates identified by transcriptome analysis have been evaluated for their role in phloroglucinol export. Efforts continue to identify the protein responsible for phloroglucinol export.